

62 1) Publication number:  
-2- BASIC DOC. Office européen des brevets C12N15/66

**O 163 406**  
**A1**

(12)

## EUROPEAN PATENT APPLICATION

(21) Application number: 85302744.9

(22) Date of filing: 18.04.85

(51) Int. Cl.<sup>4</sup>: **C 12 N 15/00**  
**C 12 P 19/34, C 12 N 1/20**  
**C 12 P 21/02, C 07 K 7/40**

(30) Priority: 01.05.84 CA 453270

(43) Date of publication of application:  
04.12.85 Bulletin 85/49

(84) Designated Contracting States:  
BE CH DE FR GB IT LI NL SE

(71) Applicant: Canadian Patents and Development Limited  
Société Canadienne des Brevets et d'Exploitation  
Limitée  
275 Slater Street  
Ottawa Ontario, K1A 0R3(CA)

(72) Inventor: Garvin, Robert T.  
33 DeForest  
Toronto Ontario M6S 1J1(CA)

(72) Inventor: Shen, Shi-Hsiang  
15 Rockford Drive Apt. 1105  
Willowdale Ontario M2R 3A3(CA)

(72) Inventor: James, Eric  
3303 Don Mills Road Apt. 2903  
Willowdale Ontario M2J 3A3(CA)

(74) Representative: Burford, Anthony Frederick et al.  
W.H. Beck, Greener & Co. 7 Stone Buildings Lincoln's Inn  
London WC2A 3SZ(GB)

(54) Novel polypeptide expression method.

(57) The stability of polypeptides, such as proinsulin, somatostatin and interferon, produced in microorganisms transformed by cloning vehicles containing a DNA sequence coding for the polypeptide, such that the polypeptide may be recovered as a product of the microorganism, is significantly improved by inserting into the cloning vehicle a DNA fragment which codes for tandemly-linked multiple copies of the polypeptide gene joined by linking sequences which code for easily-cleavable amino acid sequences. Stop and start codons are absent between the ends of the fragment, so that the whole fragment is expressed. High yields of the multiple copy (polymeric) product are obtained and may be cleaved at the contrived joins to provide single polypeptides. Novel plasmids for the specific example of human proinsulin are described.

EP 0 163 406 A1



NOVEL POLYPEPTIDE EXPRESSION METHOD

The present invention relates to the expression of polypeptides from microorganisms.

5 Bacteria, in common with mammalian and other eucaryotic cells, possess a proteolytic system for the selective degradation of abnormal or no longer needed polypeptides. In the natural course of events, abnormalities may arise by either mutation or biosynthetic error. Alternatively, a normal  
10 polypeptide may be needed in the cell only at a specific time to carry out a specific vital function while, at any other time, the presence of that polypeptide is deleterious to the cell, so that, once the polypeptide has carried out its function, it is  
15 selectively destroyed. These operations imply a complex network of interacting regulatory and structural elements, and, indeed, in Escherichia coli, the best studied bacterial system, eight different endoproteases have so far been described which  
20 function, individually or in concert, in this selective degradation system.

The introduction of foreign genes into a microorganism often has inadvertently activated this proteolytic system, thereby inhibiting the large scale  
25 production of a desired gene product by the practice of genetic engineering. This problem is particularly acute when genes coding for eucaryotic peptides are introduced into the bacteria. The strategy for circumventing this problem has been to fuse the  
30 nucleotide sequence coding for the desired gene product to at least part of a structural gene, or carrier protein gene, whose product is known to be constitutively stable.

35 The tandem arrangement of desired gene product and carrier protein results in the expression from the

organism of a hybrid polypeptide which is stable, provided that the carrier protein is large enough to identify the entire hybrid polypeptide as both native and constitutively stable. The prior art difficulty of direct expression of eucaryotic polypeptides and the necessity to use a carrier protein to achieve expression is well recognized and has been described in a number of scientific papers and published patent applications and patents, for example, in U.S. Patent No. 4,342,832. A typical patent which describes the use of this technique, for the production of somatostatin, is U.S. Patent No. 4,366,246.

A disadvantage to this approach, however, is that the desired product constitutes only a small portion of the hybrid polypeptide, thereby decreasing significantly the theoretical yield, and necessitating further processing to split off the native carrier protein and to purify the desired polypeptide product. Efforts to decrease the native carrier moiety to a small portion of the hybrid product in an attempt to improve the yield, however, render the expressed hybrid polypeptide unstable.

The symbols, abbreviations and some of the terms used herein have the following meanings:

25	DNA	- deoxyribonucleic acid
	A	- Adenine
	T	- Thymine
	G	- Guanine
	C	- Cytosine
30	Tris	- 2-Amino-2-hydroxy-ethyl-1,3-propane diol
	EDTA	- Ethylene diamine tetraacetic acid
	ATP	- Adenosine triphosphate
	TTP	- Thymidine triphosphate
35	GTP	- Guanosine triphosphate
	T4	- Bacteriophage specific to <u>E.coli</u>
	Nucleotide	- Nucleoside phosphate

Codon                    - Three nucleotide sequences specifying  
                             a particular amino acid

Oligonucleotide- Polymer consisting of a number of  
                             nucleotides

5     Plasmid               - Self-replicating, extrachromosomal  
                             genetic element

10       In accordance with the present invention, it has  
now surprisingly been found that this prior art  
problem can be satisfactorily overcome and the  
natural, selective proteolytic mechanisms of the  
microorganisms can be circumvented to achieve high  
yield production of a desired eucaryotic polypeptide.  
This is achieved by tandemly-linking a plurality of  
polypeptide genes through easily-cleavable amino  
15       sequences, incorporating the resulting DNA construct  
into a cloning vehicle, and transforming a  
microorganism with the cloning vehicle.

20       In accordance with the present invention,  
therefore, there is provided a novel DNA fragment  
having an oligonucleotide sequence coding for a  
polypeptide composed of repeating units of a desired  
product of known amino acid sequence separated by an  
oligonucleotide coding for an easily-cleavable amino  
acid sequence, wherein stop and start codons are  
absent between the ends of the fragment.  
25

30       By providing a DNA fragment composed of repeating  
units coding for a desired product of known amino acid  
sequence and separated by oligonucleotides coding for  
easily-cleavable amino acid units, a replicable  
cloning vehicle can be provided which is capable of  
stably expressing a product in a microbial organism.  
Once the product has been expressed during growth of  
the microorganism, it may be isolated from the cell  
culture, and the desired polypeptide product is  
35       obtained from the isolated product by cleaving the  
repeating units at the cleaving sites to form a  
plurality of individual units of the desired product.

Since multiple copies of the desired polypeptide product are expressed from the microorganism, the yield of product is considerably enhanced with respect to the prior art expression procedure discussed above.

5       The present invention has general applicability to the enhancement of stability of polypeptides in microorganisms and may be used to produce a wide variety of polypeptide products, comprising hormone polypeptides, including human and animal source  
10       polypeptides, for example, human proinsulin, human growth hormone, somatostatin and human interferon, and viral proteins for use in vaccines.

Essential to the present invention is the formation of a DNA fragment which contains at least  
15       two units each coding for a desired product of known amino acid, sequence, separated by a joining unit whose product is readily cleaved. The number of repeating units which is required to achieve stability depends on the desired product, while the nature of  
20       the joining unit and the cleaving agent used for cleaving also depend on the desired product.

The term "stability" as used herein with respect to the expression products refers to a material that has a half-life of sufficient duration to enable  
25       extraction thereof to be effected. It is preferred to provide by this invention products having a half-life greater than about 200 minutes.

The specific embodiment of the invention relates to the expression of human proinsulin, i.e. the  
30       precursor of insulin containing the C-chain in addition to the A and B chains, but the principles thereof are widely applicable to any polypeptide, as discussed above.

In the following description, reference is made  
35       to the accompanying drawings, in which:

Figure 1 is a schematic illustration of the formation of recombinant plasmids in accordance with this invention;

Figure 2 is a derivation and restriction site map of plasmid plac 239-3PI;

Figure 3 is a derivation and restriction site map of plasmid pTac/5PI;

Figure 4 illustrates the sequence of steps to form plasmid pAT/PIR;

Figure 5 is a derivation and restriction site map of plasmid pAT/PIR;

Figure 6 illustrates the sequence of steps to form plasmid plac 504/PI, and formation of plac 239-PI and pTac/PI therefrom;

Figure 7 is a derivation and restriction site map of plasmid plac 504/PI;

Figure 8 illustrates formation of plasmid plac 239-PI from plac 504/PI; and

Figures 9, 10 and 11 are photographs of electrophoresis experiments conducted on various products produced in accordance with this invention.

In the utilization of the present invention to effect the production of human proinsulin in accordance with the preferred embodiment hereof, a DNA fragment is formed containing a plurality of sequences coding for human proinsulin separated by oligonucleotide sequences coding for easily-cleavable units, which, in the final product, permit ready separation of the individual proinsulin peptides one from another. Sequences coding for stop and start signals are absent from the fragment between its ends. The DNA fragment is inserted into a replicable cloning vehicle which is capable of expressing the fragment in a microbial organism.

The preferred method of constructing the multiple proinsulin gene and inserting the same into a cloning vehicle, in both a fused and an unfused configuration,

is illustrated in Figure 1. In the fused configuration, the human proinsulin coding sequence is joined to the 3'-side of a fragment containing the lac promoter and encoding the first 80 amino acids of the N-terminus of  $\beta$ -galactosidase. In the unfused configuration, the proinsulin coding sequence is linked directly to a fragment containing the Tac promoter, followed by a bacterial Shine-Delgarno sequence. It has been found that the yield of expressed polypeptide, in terms of grams per cell of microorganism, is significantly greater in the case of the fused configuration, as compared with the unfused configuration.

In both configurations, the single gene human proinsulin is too unstable to be isolated as a product from the microorganism but, as additional human proinsulin coding sequences are added to the genetic construct by the method of Figure 1, the resultant multiple domain polypeptide becomes increasingly stable, approaching normal stability at three tandemly-linked proinsulin domains in the unfused configuration, and at two tandemly-linked proinsulin domains in the fused configuration. As increasing numbers of proinsulin coding sequences are tandemly linked, a maximum number of coding sequences is reached, beyond which the yield of polymer declines. For example, increasing the number of tandemly-linked units in the unfused configuration for proinsulin beyond six, results in a decreased amount of polymeric product being produced by the cell. It is preferred to utilize 4 to 5 proinsulin coding sequences in the unfused configuration and 3 to 4 proinsulin coding sequences in the fused configuration.

The oligonucleotide sequence for the linking unit or domain junction in the embodiment illustrated in Figure 1 is represented as follows:



Proinsulin gene-Arg Arg Asn Ser Met-Proinsulin gene  
AGG CGC AAT TCT ATG

5 The amino acid domain junction is easily cleavable, by reaction with cyanogen bromide, into single proinsulin peptides. This domain junction and its manner of cleaving are but one example of a variety of linking units and modes of cleaving which may be adopted.

10 For those polypeptides which contain no methionine group, cyanogen bromide cleavage may be used to produce the native material directly, provided that a methionine codon constitutes the peptide coding junction, while for those polypeptides which do contain the methionine group, the design of a particular amino acid sequence positioned between  
15 desired polypeptide segments to provide an easily-cleavable site may be effected by genetic engineering methods. For example, polypeptides which do not contain an aspartyl-proline bond may be engineered to contain such a bond between each unit of  
20 the multi-coding sequence construct, so that recovery of the native material from the isolated multi-domain polypeptide may be achieved following cleavage by acids, as described by Jauregui-Adell and Marti, Anal. Biochem. 69, 468 (1975).

25 In general, enzymatic methods or chemical reactions which are amino acid residue-sequence specific may be used to effect cleavage of linking groups between the desired polypeptide genes.

30 The authentic human proinsulin molecule contains no methionine residues, so that the cyanogen bromide treatment cleaves a multi-domain proinsulin molecule into several proinsulin analog moieties along with the C-terminal authentic proinsulin unit. This results from the designed placement of methionine codons at  
35 the 5'-end of each of the proinsulin coding sequence units.

The individual proinsulin units derived in this manner may be further processed into authentic human insulin by digestion with trypsin and carboxypeptidase B, or by any other convenient procedure.

5       The formation of a recombinant plasmid containing an oligonucleotide sequence coding for multiple copies of human proinsulin leads to a considerable increase in yield of proinsulin and proinsulin analogs, when compared with the expression in the prior art of the  
10       hybrid molecule containing the  $\beta$ -galactosidase gene.

As seen in Figure 1, the preferred sequence to form the novel plasmids pTac/2PI and plac 239/2PI, and other multiple PI unit plasmids especially the expression plasmids pTac/5PI and plac 239/3PI which is  
15       further engineered as illustrated in Figure 8 to form the preferred expression plasmid plac-3PI, starts from the plasmid pAT/PIR which contains an oligonucleotide sequence between the EcoRI and BamHI restriction sites coding for human proinsulin. The derivation from the  
20       known plasmids pBCA4 of the plasmid pAT/PIR, which is also a novel plasmid, is illustrated in Figure 4. The pTac and plac 239 fragments are derived from plasmids pTac/PI and plac 239/PI respectively, which in turn are both derived from another novel plasmid, plac  
25       504/PI, as shown in Figure 6. The derivation of plasmid plac 504/PI from known plasmids is also shown in Figure 6.

It is not essential to obtain the pTac and plac vectors from the plasmids pTac/PI and plac/PI i.e.  
30       plasmids containing a single copy of the proinsulin gene. They may be derived from any other convenient plasmid which contains the Tac and lac promotor respectively, and which can be cleaved at EcoRI and Bam HI sites to form the vector for ligation with the  
35       multiple proinsulin gene fragment. Similarly, it is not essential to form the plasmid plac 504/PI, i.e. also containing the single copy of the proinsulin

gene, as an intermediate, but any other convenient intermediate plasmid may be employed. The particular single copy proinsulin gene plasmids here embodied were formed by the inventors during the experimentation which led to this invention in an attempt to provide constructs from which proinsulin would be stably expressed. As noted earlier, this was not found to be possible with the single copy gene.

Specific experimental details for the formation of the various plasmids set forth in the drawings and disclosed above are discussed in the Examples below.

Detailed derivations and major restriction site maps for the novel plasmids plac 239-3PI, pTac/5PI, pAT/PIR and plac 504/PI appear as Figures 2, 3, 5 and 7 respectively. E.coli JM103 modified by each of the plasmids plac 239-3PI and pTac/5PI, have been deposited on July 6, 1984 with the American Type Culture Collection located at Rockville, Maryland, U.S.A., and have been accorded the following accession Nos.:

Organism E.coli/plasmid plac 239-3PI - ATCC 39760

Organism E.coli/plasmid pTac/5PI - ATCC 39759.

The invention is illustrated further by the following Examples.

Example 1

This Example describes the formation of novel plasmids containing multiple units of the human proinsulin gene.

(a) Preparation of pAT/PIR

The plasmid pAT/PIR was prepared following the sequence outlined in Figure 4 and the restriction map for the plasmid appears as Figure 5. The starting plasmid pBCA4 contains a nucleotide sequence coding for human proinsulin between its EcoRI and BamHI restriction sites. However, if the EcoRI-BamHI fragment of this plasmid is used directly, the wrong reading frame results and hence must be modified as

described below. Plasmid pBCA4 is described in Narang et al, Gene, 17, 279 (1982) and was obtained from Professor R. Wu, an author of that paper of Cornell University, Ithaca, N.Y., U.S.A.

5        The plasmid pBCA4 was cut with FokI and BamHI and the FokI-BamHI fragment was isolated. Two synthetic oligonucleotides were created and ligated with the FokI-BamHI fragment to extend the 5'-end of the PI  
10       PI sequences being designated PIR. The synthetic nucleotide sequences were generated chemically and comprised the sequences:

5' AATTCTATGTTTGTCAAT       3'  
3'       GATACAAACAGTTAGTCG       5'

15       The starting plasmid pAT153 is a high copy variant of the well-known plasmid pBR322 with the so-called "poison" sequences removed and is described by Twigg et al, Nature, 283, 216 (1980). The plasmid pAT153 was obtained from Professor O. Smithies, Dept.  
20       of Genetics, University of Wisconsin - Madison, Madison, Wisconsin, U.S.A. The plasmid pAT153 was cut with EcoRI and BamHI and the vector isolated. The isolated vector was ligated to the EcoRI-BamHI (PIR) fragment, to form the new plasmid pAT/PIR.

25       (b) Preparation of Multiple Copies of Proinsulin Gene

      The sequence of operations required to form multiple copies of the proinsulin gene is outlined in Figure 1. Synthetic oligonucleotides (A) and (B) were prepared chemically to provide the following  
30       sequences:

(A) 5' CCTCTACCAGCTGGAGAACTACTGCAACAGGCGC       3'  
(B) 3'       ATGGTCGACCTCTTGATGACGTTGTCCGCGTTAA       5'

200 p.moles of oligonucleotide B and 200 p.moles of oligonucleotide A having its 5'-end labelled with  
35       <sup>32</sup>P-ATP, were mixed in 30 ul of 66 mM Tris-HCl (pH 7.6), 6.6 mM MgCl<sub>2</sub> and 10 mM dithiothreitol, and heated at 80°C for 15 minutes to anneal the

oligonucleotides to form a part of the human proinsulin coding sequence beginning at the SfaNI cut site near the 3'-end of the coding sequence and terminating with the last proinsulin codon (AAC), followed by the additional sequences:

5' AGG CGC 3'

3' TCC GCG TTAA 5'

These additional sequences of the synthetic oligonucleotides encode two arginine residues and provides a sticky end for ligation to an EcoRI sticky end. The ligation destroys the EcoRI site because of the CG base pair (boxed in Figure 1).

10  $\mu$ g of pAT/PIR, prepared as described in (a) above, was digested for 1 hour at 37°C with 40 units each of EcoRI and BamHI in 40  $\mu$ l of digestion buffer. The digested DNA was dephosphorylated by digestion at 37°C for 30 minutes with 4 units of calf intestine alkaline phosphatase in 200 mM Tris-HCl, pH 8.0. The proinsulin gene fragment (PIR) was isolated from 1% agarose gel by phenol extraction. The isolated fragment was further digested for 3 hours at 37°C with 6 units of SfaNI in 40  $\mu$ l of digestion buffer and the EcoRI-SfaNI fragment of PIR was isolated.

100 p.moles of the annealed oligonucleotides (A) and (B) and approximately 4 p.moles of the isolated PIR EcoRI-SfaNI fragment were ligated for 14 hours at 15°C in 30  $\mu$ l of 66 mM Tris-HCl (pH 7.6), 6.6 mM MgCl<sub>2</sub>, 10 mM dithiothreitol and 1 mM ATP using 20 units of T4 DNA ligase. As a result of dephosphorylation of the proinsulin gene fragment prior to SfaNI cleavage, and phosphorylation of only oligonucleotide (A), as described above, the reaction was unidirectional, resulting in ligation of the proinsulin gene fragment PIR to the annealed oligonucleotide. The ligation reaction product was electrophoresed on 1.5% agarose gel and the resultant

proinsulin gene analog construct (PI Analog) was isolated from the gel.

5 The isolated proinsulin gene analog sequence (approximately 3 p.moles) was phosphorylated with ATP and T4 polynucleotide kinase. 10  $\mu$ g of plasmid pAT/PIR, prepared as described in (a) above, was digested for 1 hour at 37°C with 40 units each of EcoRI and BamHI. Approximately 3 p.moles of the PIR proinsulin gene sequence were isolated from agarose  
10 gel. The isolated proinsulin gene sequence and phosphorylated proinsulin gene analog sequence were ligated at 37°C for 3 hours in 30  $\mu$ l of ligation buffer containing 20 units of T4 ligase, 40 units of EcoRI and BamHI.

15 The ligation mixture was run on 1.0% agarose gel and, after autoradiography, the dimer, i.e. two proinsulin coding sequences, and trimer, i.e. three proinsulin coding sequences, were isolated from the gel by phenol extraction. The tetramer, pentamer and  
20 hexamer, i.e. four, five and six proinsulin coding sequences respectively, were obtained by further ligating the isolated fragments with additional proinsulin gene analogs following the general procedure described above.

25 (c) Preparation of pTac and plac 239 Recombinants containing Multiple PI Units

The various PI fragment constructs, containing from two proinsulin coding sequences up to six proinsulin coding sequences, were inserted into the  
30 vectors pTac and plac 239 by ligation, as generally described in (b) above. The pTac and plac 239 vectors were formed by digestion with EcoRI and BamHI of pTac/PI and plac 239/PI respectively, which, in turn, were formed following the procedures which are  
35 outlined below in sections (d) and (e). By inserting a proinsulin gene trimer into the plac 239 vector and a proinsulin gene pentamer into the pTac vector, there

were obtained the fused and unfused multiple proinsulin gene plasmids plac 239/3PI and pTac/5PI respectively. The derivation and main restriction site map for the latter new plasmid appears as Figure 3.

In similar manner, other multiple proinsulin gene plasmids, including plac 239/4PI, plac 239/5PI, pTac/3PI, pTac/4PI, and pTac/6PI were constructed from the various multimer proinsulin genes and the plac 239 and pTac vectors. The various constructs, containing from 1 to 6 copies of the proinsulin coding sequence, were confirmed by restriction mapping and DNA sequence analysis.

(d) Preparation of plac 239-PI

The derivation of plasmid plac 239-PI is illustrated in Figure 6 and was prepared from plasmid plac 504/PI, as shown in detail in Figure 8. The formation of plasmid plac 504/PI is described in more detail below in section (f). The plasmid plac 504/PI has a lac promoter, the  $\beta$ -galactosidase gene (z-gene) complete to the EcoRI site near the carboxy terminal of the gene with the human proinsulin gene insert of plasmid pBCA4 fused at this EcoRI site, so as to remain in reading frame, such that the translation of the z-gene continues directly to code for proinsulin.

Referring to Figure 8, plasmid plac 504/PI was digested with MstII and the resulting sticky ends were filled in with dTTP and dGTP. The 5'-T's were then removed by digestion with mung bean nuclease, the fragment cut with EcoRI to remove the Mst II/EcoRI segment of the z-gene, the sticky ends of EcoRI were filled in with dATP and dTTP, and the plasmid was blunt-end ligated to establish a new EcoRI site, the resulting plasmid being designated as plac 239/PI. The plasmid was cut with EcoRI at the new site, the fragment EcoRI tails were digested with mung bean nuclease, and the resulting fragment blunt-end ligated to form the plasmid plac 239-PI in which the

correct PI reading frame was restored. In order to restore the correct PI reading frame in other fused plasmids, for example, plac 239/3PI, the EcoRI tails should also be removed by the same procedure described above, to form the corresponding expression plasmid plac 239-3PI. The derivation and main restriction site map for plac-3PI appears as Figure 2.

(e) Preparation of pTac/PI

The preparation of plasmid pTac/PI is illustrated in Figure 6 and was effected from plac 504/PI and from plasmid pDR540. The plasmid pDR540 is described by Russell et al in Gene, Vol. 20, 231 (1982) and has the following features: a HindIII site upstream from the promoter, a -35 region from trp, the -10 operator, Shine-Delgarno, and Shine-Delgarno-to-start spacing regions of lac, with a BamHI site positioned at the start. The plasmid pDR540, purchased from P-L Biochemicals, Milwaukee, Wisconsin, U.S.A., was cut with BamHI and HindIII, the sticky ends removed with mung bean nuclease and the isolated Tac promoter was inserted by blunt-end ligation into the isolated HindIII-EcoRI fragment of plasmid plac 504/PI which contains the PI gene, by first digesting plasmid plac/PI with HindIII and EcoRI and then filling in the ends with DNA polymerase I. In this way, the Tac promoter of the plasmid pDR540 is substituted for the wild-type lac promoter and the z-gene portion of plac 504/PI, thereby preserving the sequence, spacing and properties of the hybrid promoter, but with an EcoRI sequence appropriately located.

(f) Preparation of plac 504/PI

Plasmid plac 504/PI was prepared following the sequence of steps set forth in Figure 6 and the derivation and main restriction sites for the plasmid are illustrated in Figure 7.

The initial step in the formation of plasmid plac 504/PI was the formation of plasmid plac 19 from known



plasmids pMC1396 and M13mp7. The plasmid pMC1396 is described by Casadaban et al in J.Bact., Vol. 143, 971, (1980) while the plasmid M13mp7 is described by Messing et al in Nucleic Acids Research, Vol. 9, 309 (1981). Plasmid M13mp7 was purchased from BRL Inc., Gaithersburg, MO, U.S.A. and plasmid pMC1396 was a gift of M. Casadaban of Cambridge, MA, U.S.A.

Plasmid pMC1396 was digested with SmaI, treated with Bal 31 and ligated with HindIII linkers to form plasmid plac 9. Plasmid pM13mp7 was digested with AvaII and ligated to a HindIII linker to form plasmid plac 1. Both plasmid plac 9 and plac 1 were digested with HindIII and ligated to form the plac 19 plasmid.

Plasmid pBCA4, containing the sequence coding for human proinsulin, was digested with EcoRI and SalI and the EcoRI - SalI fragment was isolated. Plasmid plac 19 was digested with EcoRI and SalI and the resulting vector was ligated with the EcoRI-SalI fragment of plasmid pBCA4 to form plasmid plac 19/PI.

Plasmid pBGP120 contains the lac promoter and is described in Polisky et al, Proc. Nat. Acad. Sci. Vol. 73, 3900 (1976). This plasmid, obtained from Dr. R. Wu of Cornell University, Ithaca, N.Y., U.S.A., was digested with EcoRI and HindIII and the HindIII-EcoRI fragment containing the lac promoter was separated. Following digestion of the plac19/PI plasmid with EcoRI and HindIII, the resulting vector was ligated with the HindIII-EcoRI fragment of plasmic pBGP120 to obtain the plasmid plac 504/PI.

## Example 2

This Example illustrates the preparation of converted microorganisms from the novel plasmids containing multiple proinsulin genes and expression of the polyproinsulin polypeptide.

### (a) Transformation and Growth of Cells

The novel plasmids plac 239-nPI, where n is 2 to 5, and pTac/mPI, where m is 3 to 6, were used to

transform the strain of E.coli known as JM103. This strain is described by Messing et al in Nucleic Acids Res., 9, 309 (1981) and is characterized by the genotype (lac pro), thi, strA, supE, endA, sbcB, hsdR, F traD36, proAB, lacI<sup>q</sup>, Z M15. The use of this E.coli strain was for convenience only and other E.coli strains and indeed other microorganisms could have been used.

Transformed strains were grown in YT medium containing 50 µg/ml of ampicillin. YT medium contains, per litre, 8 g tryptone, 5 g yeast extract, 5 g NaCl, and 1 ml 1N NaOH (to pH 7.0 to 7.2). In order to induce the expression of the polyproinsulin polypeptide from the plasmid, isopropyl-B-D-thiogalactoside (IPTG) was added to provide a final concentration of 1 mM when the cell density had reached an optical density of 0.1, measured at a wavelength of 560 nm.

#### (b) Recovery of Expressed Polypeptide

After induction by IPTG, cell growth was continued for about 8 to 12 hours. The cells were harvested by centrifugation, washed with phosphate-buffered saline, and either analyzed directly by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis), as described by Laemmli in Nature, 227, 680 (1970), or further purified. Such further purification was effected by suspending washed cells from 3 ml of culture in 1.0 ml of T buffer (i.e. 50 mM Tris HCl at pH 7.9 containing 25% sucrose, 1% Nonidet-P40, 0.5% sodium deoxycholate and 5mM EDTA) and subjecting the cells to sonic disruption by two 60 second bursts at a wavelength of 12 µm at 0°C. The sonicated material was centrifuged for 10 minutes in an Eppendorf microcentrifuge at 4°C. The centrifuged pellets were dissolved in the sample buffer and analyzed by SDS-PAGE.

(c) Analysis of Expression From Multiple PI Gene Constructs

Electrophoretic analysis of multiply-expressed proinsulin polypeptides in the unfused configuration is shown in Figure 9. Proteins, partially purified as described above, were analyzed by 15% SDS-PAGE. Lanes 1 to 5 represent the products equivalent to 500  $\mu$ l of original culture of JM103 cells harbouring the plasmids pTac/PI, pTac/2PI, pTac/3PI, pTac/4PI and pTac/5PI, while lane M contains protein molecular weight markers.

Electrophoretic analysis of multiply-expressed proinsulin polypeptide in the fused configuration is shown in Figure 10. Lanes A, B and C represent the total cell protein equivalent to 150  $\mu$ l of original culture of JM103 cells harbouring the plasmids plac 239-PI, plac 239-2PI and plac 239-3PI while lanes D, E and F were the same products as lanes A, B and C, except that the products were partially purified, as described above. Lane M contains protein molecular weight markers.

The amount of product obtained per cell of microorganism was not determined quantitatively but was qualitatively observed to be significantly greater for the multiple gene fragments when compared with that known to be typically obtained when expressing hybrid polypeptides in the prior art.

Example 3

This Example illustrates the isolation and purification of polypeptide fractions and the formation of insulin therefrom.

(a) Recovery of Proinsulin

About 7 g of cells were suspended in T buffer using 10 ml/g and were sonically disrupted at 0°C using two 90 second bursts at a wavelength of 18  $\mu$ m. Following centrifugation at 12,000 g for 10 minutes, the pellet was suspended in 25 mM Tris-HCl, 2 M NaCl,

4 M urea at pH 8.4, using 5 ml/g of cells, resonicated and recentrifuged at 12,000 g. In order to solubilize the polyproinsulin, the pellet was resuspended in GDFT buffer (7 M guanidinium chloride, 100 mM sodium formate, pH 3.0) using 5 ml/g cells and gently stirred for 1 hour. The polyproinsulin contained in the GDFT buffer was precipitated by dilution into 6 volumes of ice-cold water, and collected by low speed centrifugation at 5,000 g. The resultant pellet contained essentially pure proinsulin polypeptide polymer, as determined by SDS-PAGE (see Fig. 11, Lane B).

Purified proinsulin polymer was dissolved in 70% formic acid using 10 ml/2 g cell equivalents, digested with cyanogen bromide at 50 mg per mg of protein for 35 hours at room temperature, and the product analyzed by SDS-PAGE. Lane A in Figure 11 shows the cleaved product of the five tandem proinsulin polypeptide. Lane M contains protein molecular weight markers. A Western blot of the SDS-PAGE gel of Lanes A and B showed the human proinsulin-sized material to be reactive with porcine anti-proinsulin sera.

#### (b) Production of Insulin

Recovered cyanogen bromide digest product was sulfitolyzed by dissolving the material in GE buffer, containing 6 M guanidinium HCl and 0.2 M ethanolamine (pH 9), at 10 mg/ml, adding  $\text{Na}_2\text{SO}_3$  and  $\text{Na}_2\text{S}_4\text{O}_6$  to concentrations of 0.4 M and 0.8 M respectively, and allowing the reaction to occur for 2 hours at room temperature. The sulfitolyzed product was precipitated by dialysis against 10 mM ammonium acetate (pH 4.5). The precipitate was dissolved at 4 mg/ml in TF buffer, containing 25% formamide and 50 mM Tris-HCl (pH 8.1) and the solution loaded onto a DEAE cellulose column in the ratio of 200 mg per 2.5x10 cm column. The products were differentially eluted by sodium chloride during application of a 0 to 500 mM

linear NaCl gradient.

5 The sulfitolyzed biosynthetic human proinsulin (BHPI-SSO<sub>3</sub>) was readily identifiable by HPLC-analysis of the salt gradient elution profile. BHPI-SSO<sub>3</sub> was collected and dialyzed at pH 4.5 to remove formamide and to precipitate the product. The pure BHPI-SSO<sub>3</sub> precipitate was dissolved in RH buffer, containing 50 mM glycine and 5 mM EDTA (pH 10.5), at 0.5 mg/ml and cooled to -2°C. Ethanethiol was added to 800 µm and 10 allowed to react for 24 hours to effect refolding of the molecule. The reaction was stopped by adjusting the pH to 7 with HCl and the reaction product was analyzed. Refolded biosynthetic human proinsulin (BHPI) constituted the monomeric component of the 15 reaction product.

The BHPI was dissolved in TC buffer, containing 100 mM Tris-HCl (pH 7.6) and 10 mM CaCl<sub>2</sub>, at 2 mg/ml, 10 µg/ml of trypsin and 40 µg/ml of carboxypeptidase B were added, and the mixture was allowed to react for 20 30 minutes at room temperature. The reaction was stopped by adding acetic acid to 1% and biosynthetic human insulin (BHI) was recovered by precipitation. The recovered yield was 98% of theoretical, based on the amount of BHPI used.

25 The BHI was considered authentic on the basis of HPLC analysis, amino acid composition, amino acid sequencing and biological activity. Human proinsulin in the form of tandemly-linked units, therefore, is stably expressed from transformed cells and human 30 insulin can be readily formed from the expressed product.

In summary of this disclosure, the present invention provides a novel procedure for expressing polypeptides from organisms, which involves providing 35 a DNA fragment containing a multiple number of oligonucleotide sequences each coding for a desired polypeptide product and tandemly-linked by

oligonucleotides coding for easily-cleavable amino  
acid groupings, forming the fragment into a  
recombinant plasmid and transforming an organism by  
the plasmid. Modifications are possible within the  
5 scope of this invention.

10

15

20

25

30

35

CLAIMS

1. A DNA fragment having an oligonucleotide sequence coding for a polypeptide composed of repeating units of a desired product of known amino acid sequence  
5 tandemly-linked by an oligonucleotide coding for an easily-cleavable amino acid sequence and having no codons for stop and start signals between the ends of said fragment.
2. The fragment claimed in claim 1 in a replicable  
10 cloning vehicle capable of expressing the fragment in a microbial organism.
3. The fragment claimed in claim 1 or 2 characterized in that the desired product is human proinsulin.
- 15 4. The fragment claimed in any one of claims 1 to 3, characterized by from 2 to 6 repeating units each coding for human proinsulin and being tandemly-linked through oligonucleotides coding for easily-cleavable amino acid sequences.
- 20 5. A recombinant plasmid, comprising a plasmid vector and an expressible DNA fragment ligated thereto, characterized in that the DNA fragment is composed of repeating units coding for a desired  
25 product of known amino acid sequence tandemly-linked by oligonucleotides coding for an easily-cleavable amino acid sequence.
6. The plasmid claimed in claim 5 characterized in that the desired product is human proinsulin, the DNA  
30 fragment is joined to the 3'-side of a lac operon fragment encoding the first approximately 80 amino acids of  $\beta$ -galactosidase, and the fragment contains from 2 to 5 tandemly-linked oligonucleotide sequences coding for human proinsulin.
- 35 7. The plasmid claimed in claim 5 characterized in that the desired product is human proinsulin, the DNA fragment is directly linked to a fragment of the cloning vector containing the Tac promoter followed by a bacterial Shine-Delgarno sequence, and the fragment

contains from 3 to 6 tandemly-linked oligonucleotide sequences coding for human proinsulin.

5 8. A microorganism which has been transformed by the plasmid claimed in any one of claims 5 to 7 for expression of said DNA fragment therefrom.

9. A method of producing polypeptides by microorganisms, characterized by:

(a) cleaving a vector at a predetermined restriction site;

10 (b) preparing an insert to the cleaved vector which has multiple copies of the gene for the polypeptide separated by oligonucleotide sequences coding for easily-cleavable amino acid sequences;

15 (c) ligating the insert to the cleaved vector to form a recombinant plasmid containing the insert;

(d) transforming a microorganism with the recombinant plasmid;

20 (e) growing said microorganism in a culture medium to effect expression of a DNA fragment containing said multiple copies of the coding sequence for the polypeptide;

(f) isolating from the microorganism a fraction constituting a polymer composed of the multiple linked copies of the polypeptide; and

25 (g) cleaving the isolated multiple copy polypeptide to single polypeptides.

10. The method claimed in claim 9, characterized in that the polypeptide is human proinsulin.

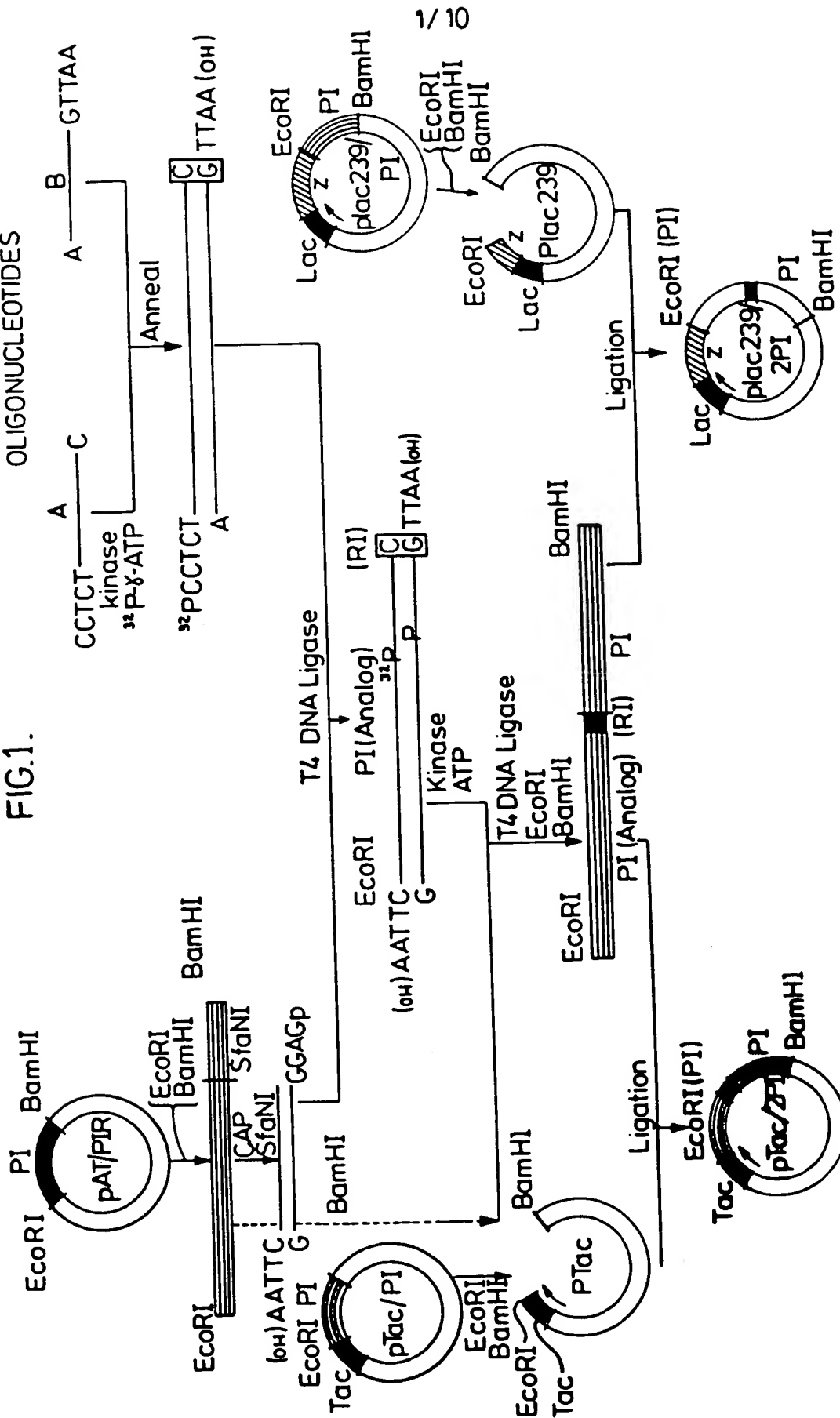
30 11. The method claimed in claim 10, characterized in that the human proinsulin is converted into human insulin.

12. The method claimed in any one of claims 9 to 11, characterized in that step (f) includes sonic disruption of cells of the microorganism separated from the culture medium.

35



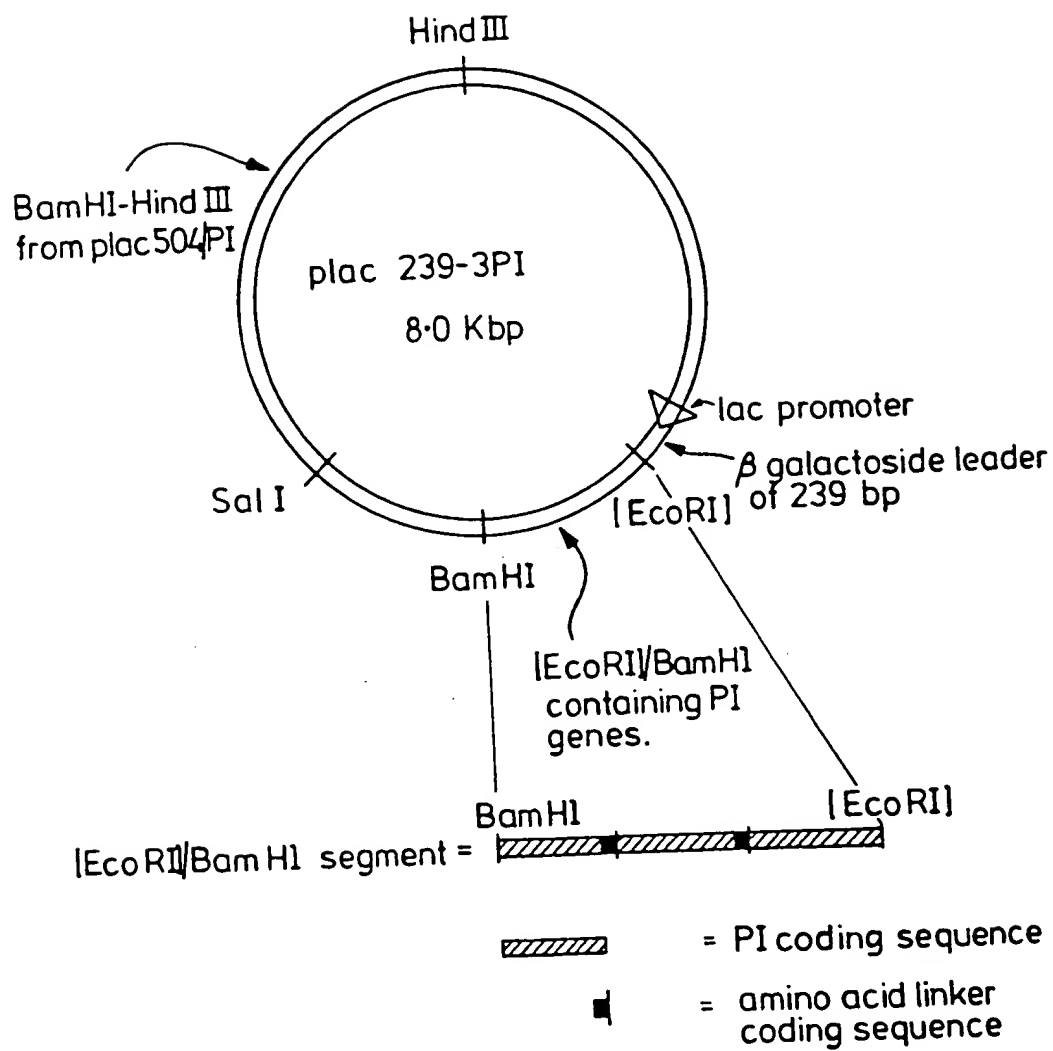
## OLIGONUCLEOTIDES





2/10

FIG. 2.

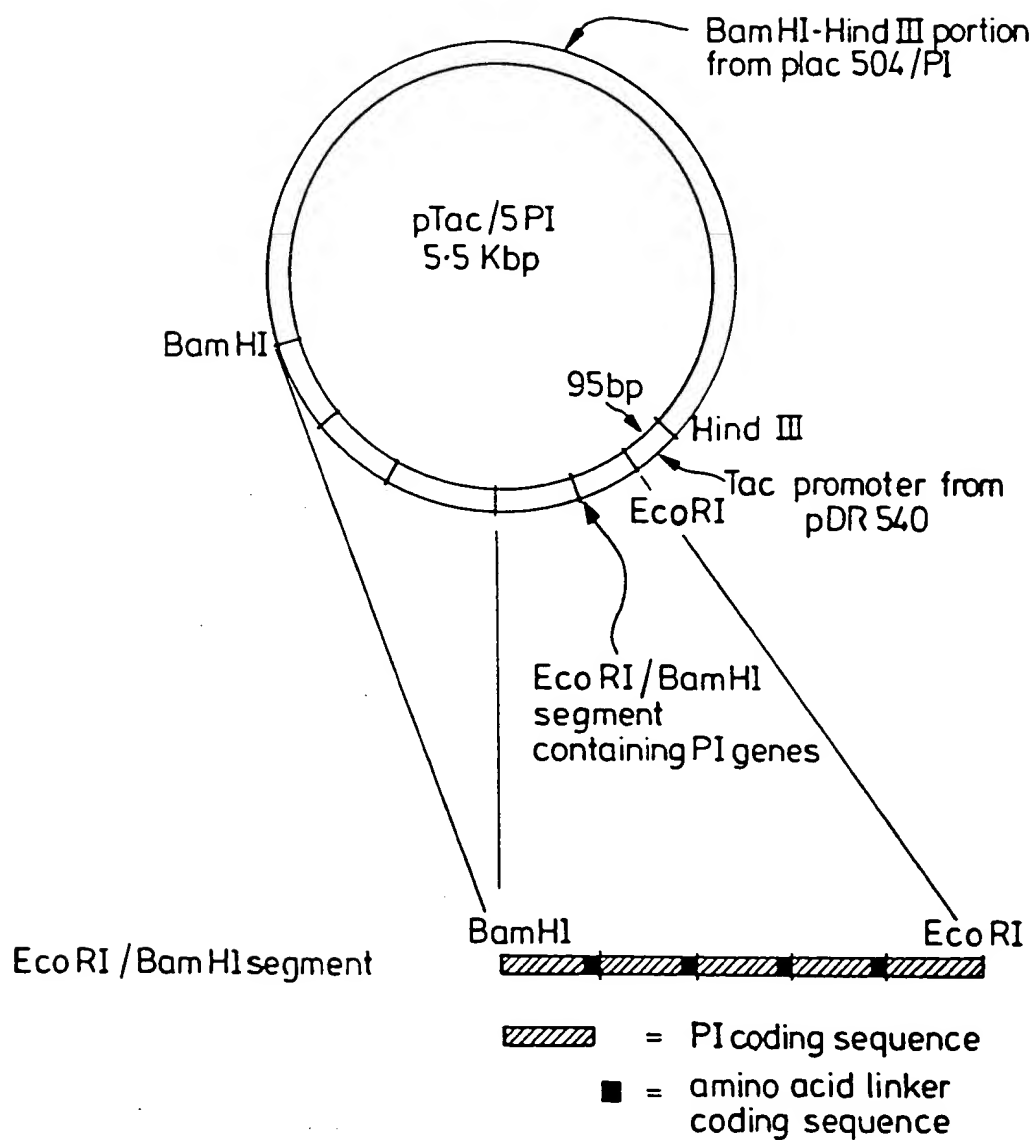




0163406

3/10

FIG. 3.





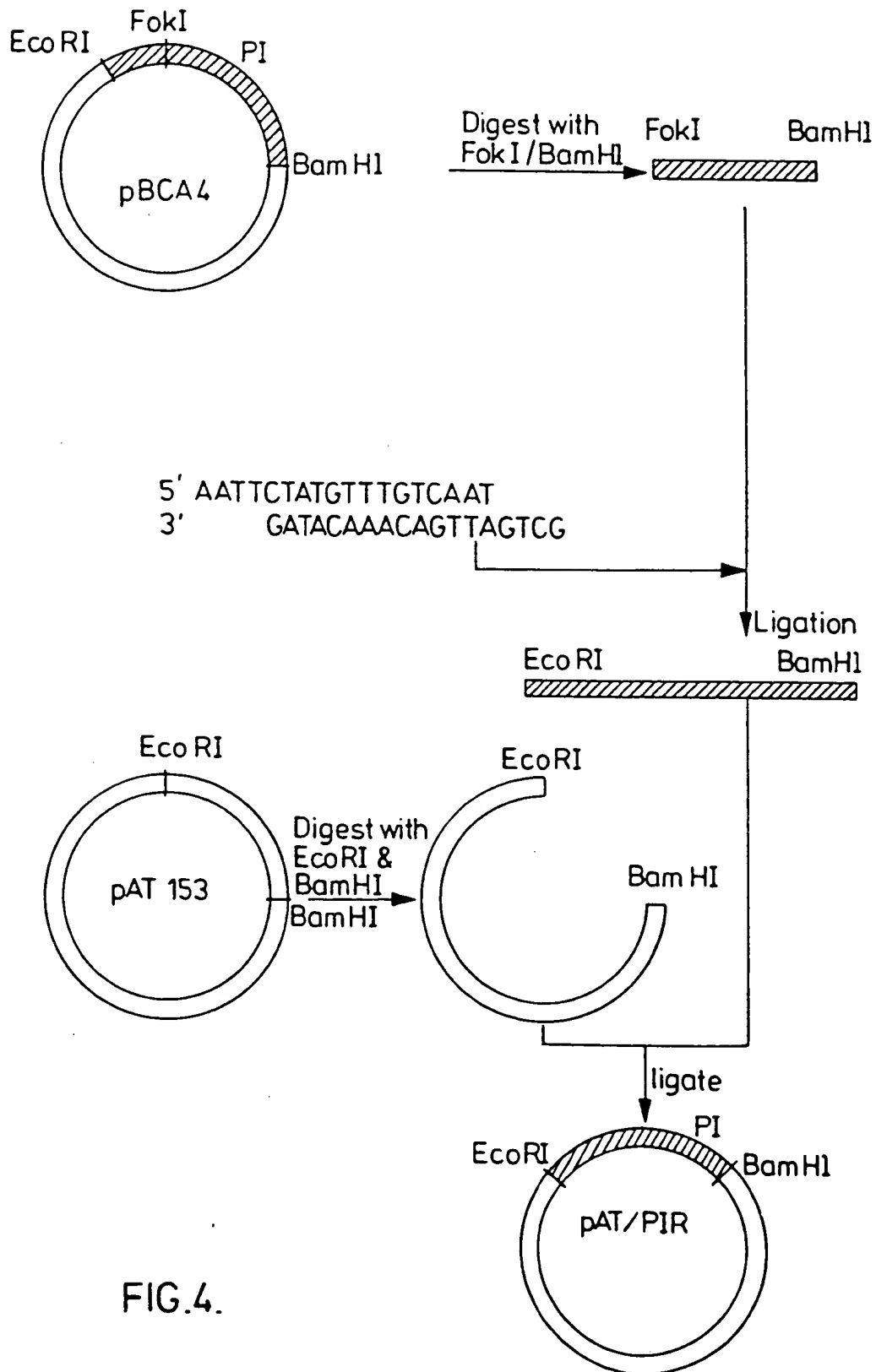


FIG.4.

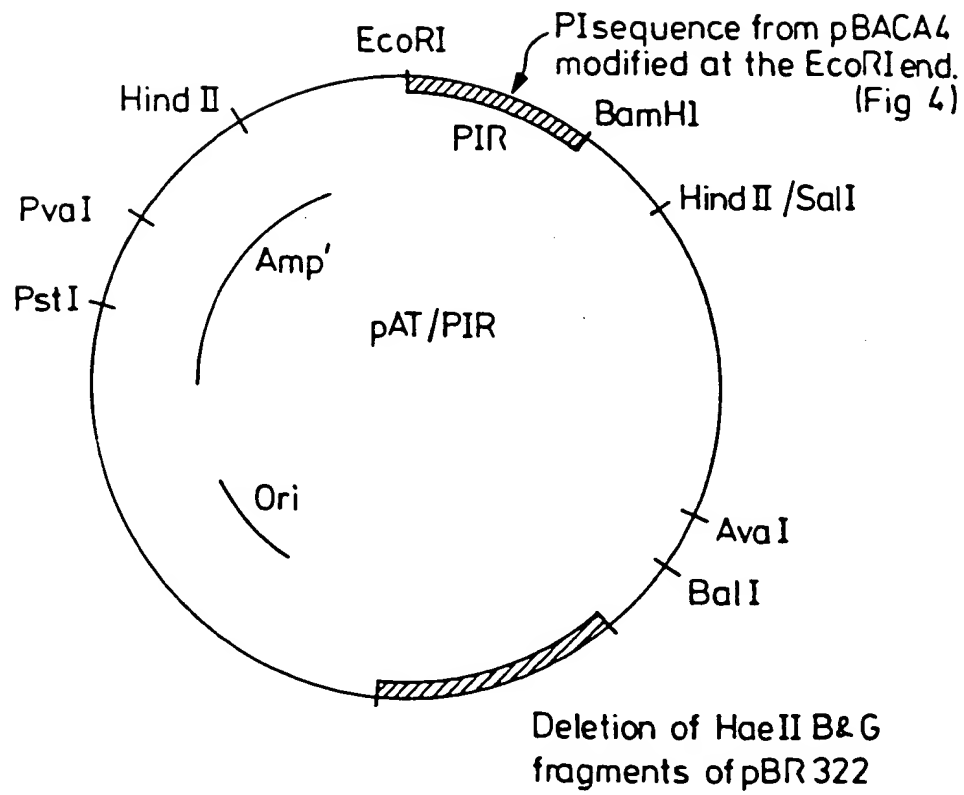




10507  
0163406

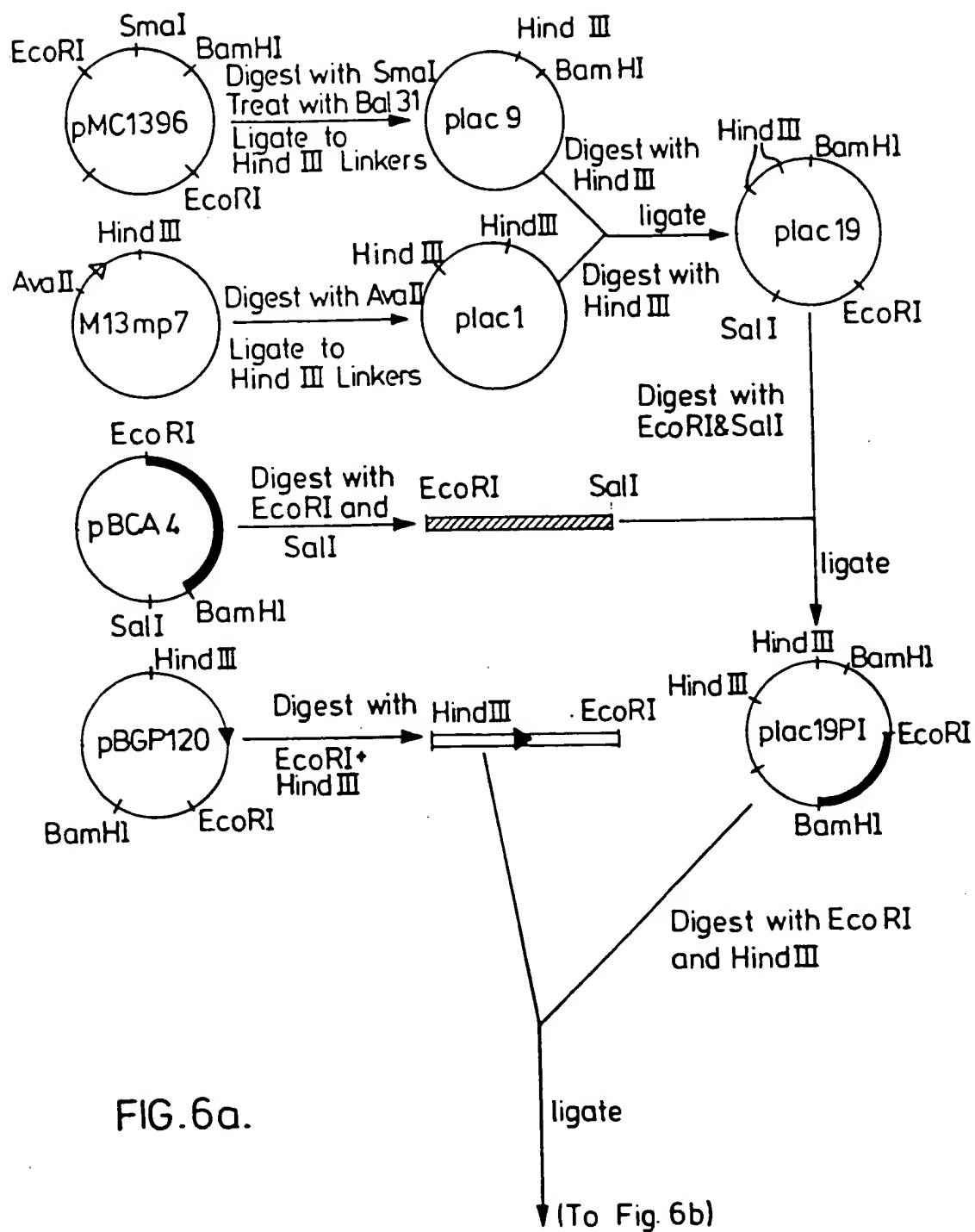
5/10

FIG. 5.





6/10





0163406

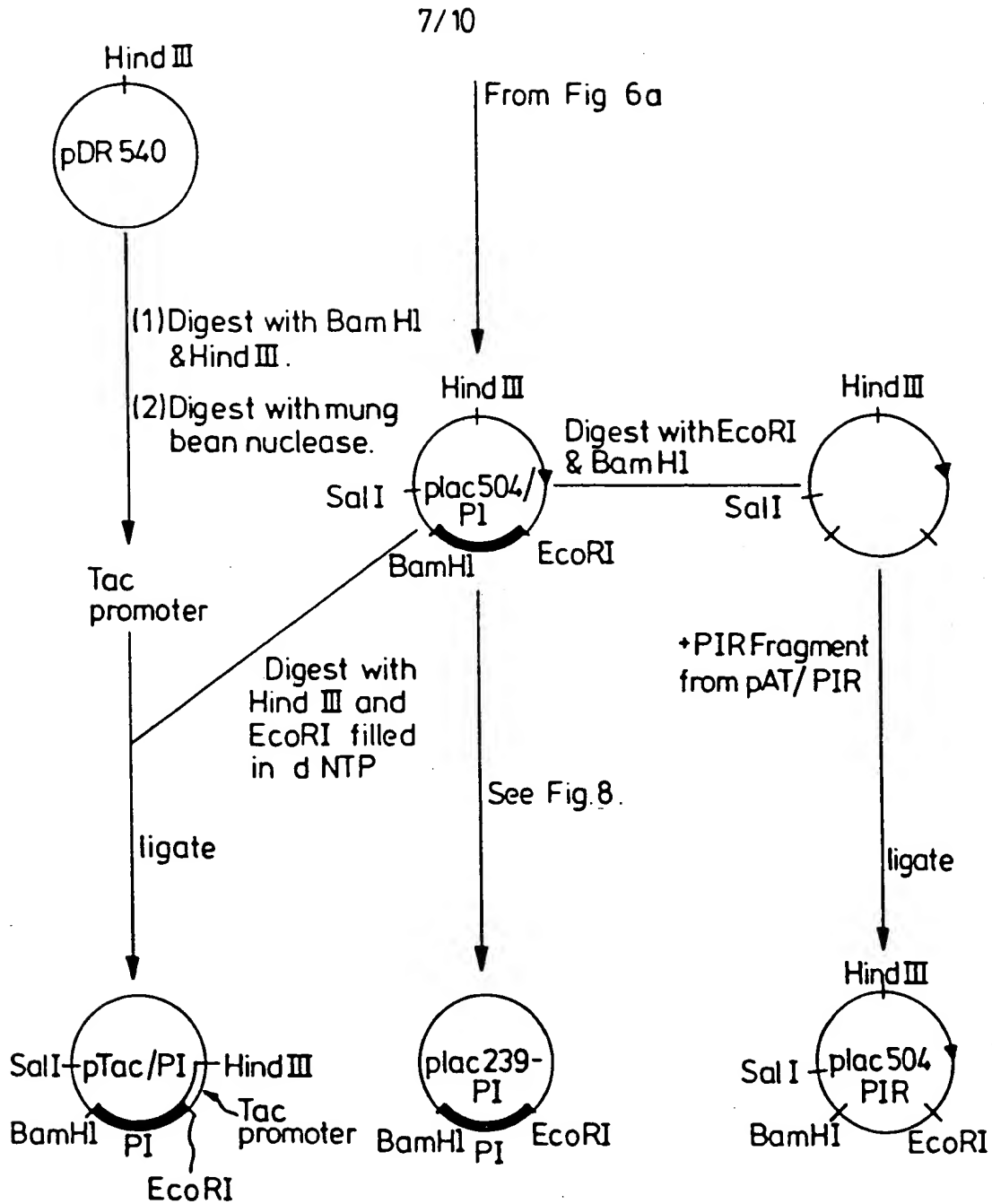


FIG.6 b.



8/10

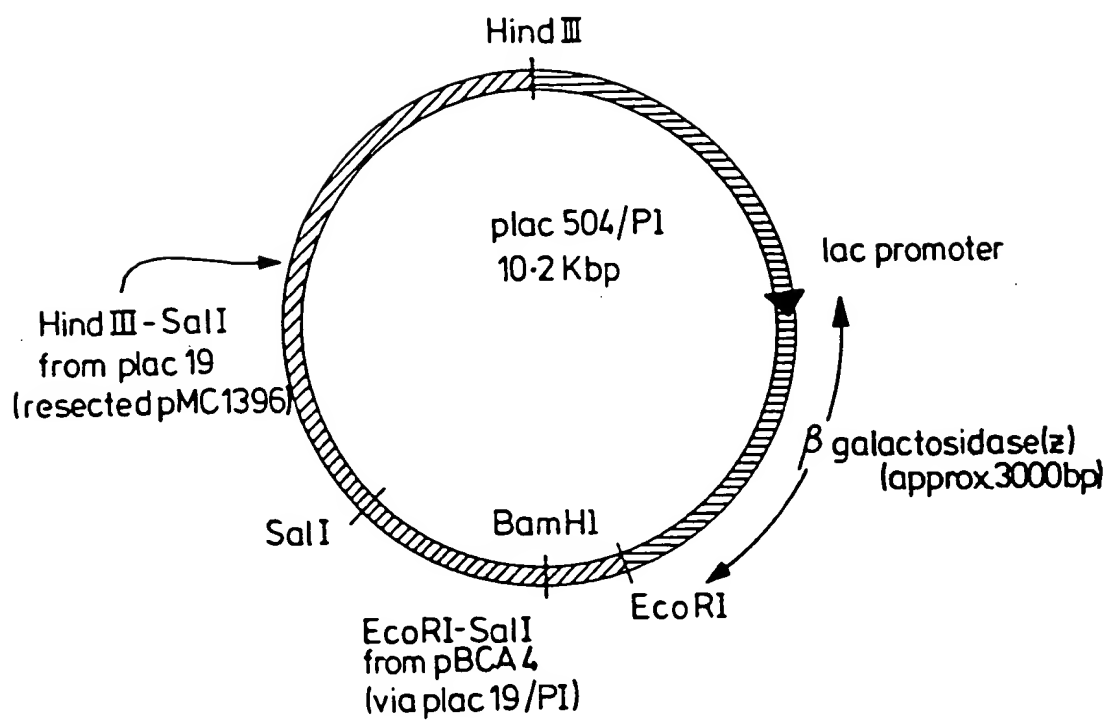


FIG.7.





9/10

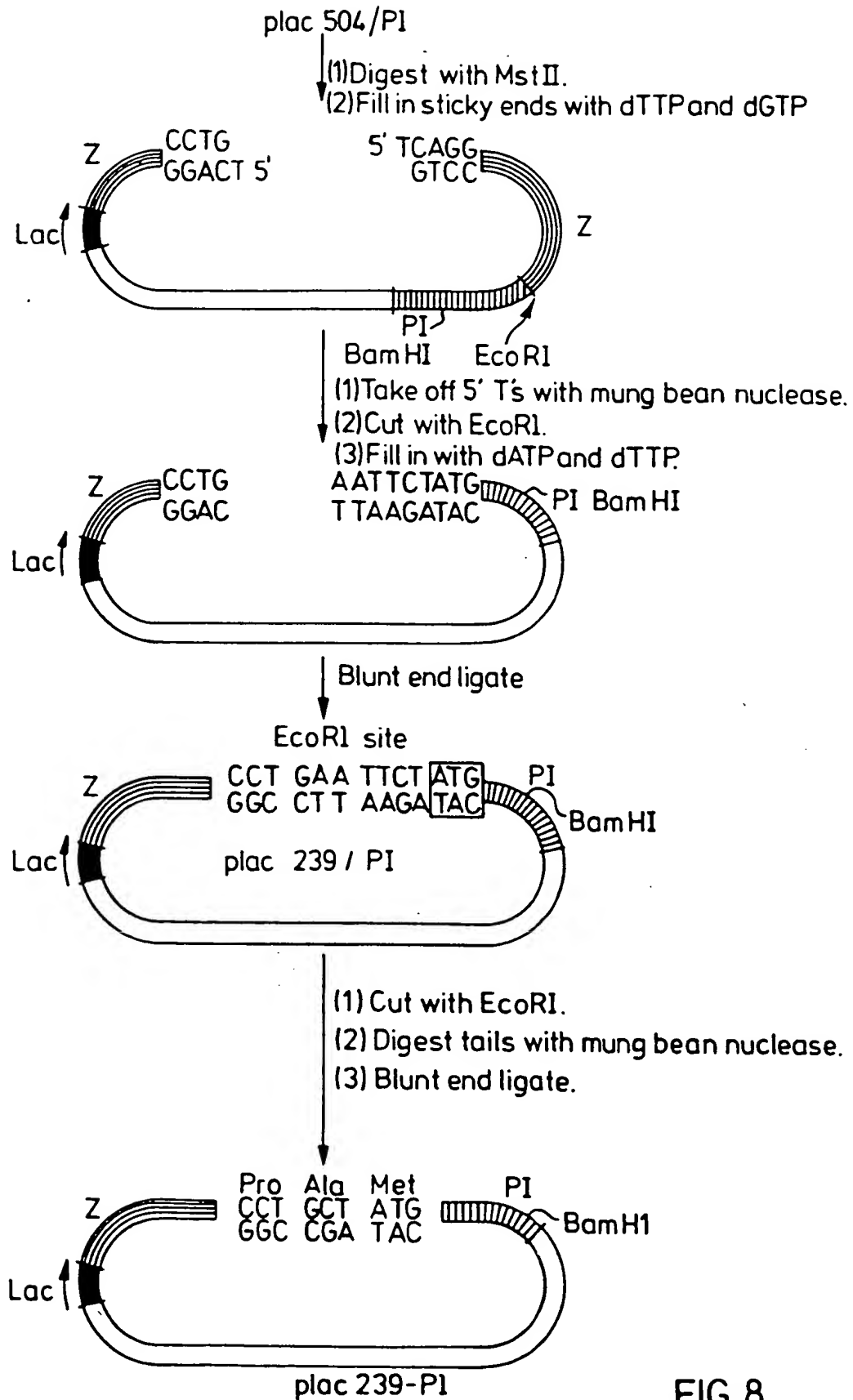


FIG.8.



0163406

10/10

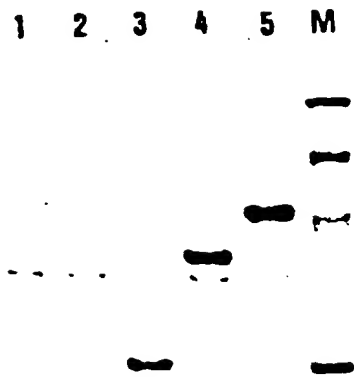


FIG. 9.

FIG. 10.

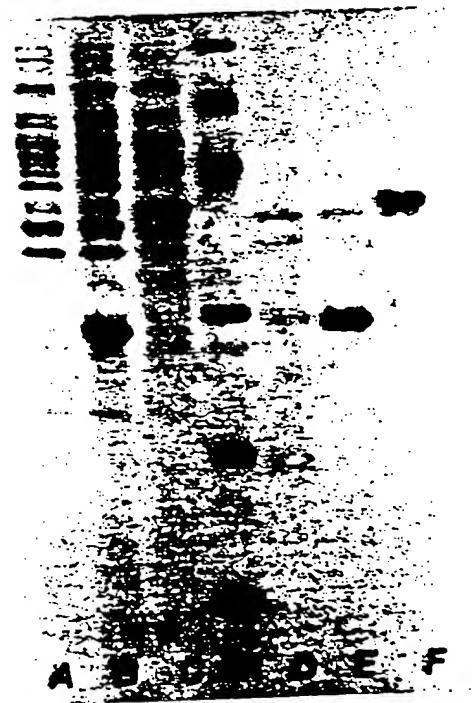


FIG. 11.





European Patent  
Office

# EUROPEAN SEARCH REPORT

01 634 06  
Application Number

EP 85302744.9

DOCUMENTS CONSIDERED TO BE RELEVANT			Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
Category	Citation of document with indication, where appropriate, of relevant passages			
A	EP - A2 - 0 040 466 (CANADIAN PATENTS AND DEVELOPMENT LIMITED) * Abstract *	1,3	C 12 N 15/00 C 12 P 19/34 C 12 N 1/20 C 12 P 21/02 C 07 K 7/40	
D,A	US - A - 4 366 246 (RIGGS) * Abstract *	1,9		
D,A	US - A - 4 342 832 (GOEDDEL et al.) * Claim 1 *	1,2		
The present search report has been drawn up for all claims			TECHNICAL FIELDS SEARCHED (Int. Cl. 4)	
			C 12 N C 12 P C 07 K	
Place of search		Date of completion of the search		Examiner
VIENNE		07-08-1985		WOLF
CATEGORY OF CITED DOCUMENTS				
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document				

**THIS PAGE BLANK (USPTO)**